Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. Max Gassmann

Arbeit unter Leitung von Dr. Markus Thiersch

Hypoxia-induced Resistance to Radiotherapy in Lung Cancer Cells is Mediated by the Erythropoietin Receptor

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Nadine Jänicke

Tierärztin

aus Berlin, Deutschland

genehmigt auf Antrag von

Prof. Dr. Max Gassmann, Referent

Prof. Dr. Carla Rohrer Bley, Korreferentin

Zürich 2017

Table of Content

1 **Summary** 1

2 **Zusammenfassung** 2

3 **Introduction** 3

4 **Materials and Methods** 5

4.1 Cell culture 5

4.2 CRISPR/Cas9 EPOR Knock Out Generation 5

4.3 Clonogenic Assay 8

4.4 γH2AX- a Marker for DNA-damage 10

4.5 RNA Extraction, cDNA Synthesis and Real-time PCR 11

4.6 Western Blot 12

5 **Results** 12

5.1 The Loss of EPOR Sensitizes A549 Lung Cancer Cells towards Gemcitabine 12

5.2 Hypoxia-inducible Resistance to Radiotherapy is EPOR Dependent 14

5.3 EPOR-dependent Radioresistance does not Depend on Protection from DNA-damage or Repair of DNA-damage 15

5.4 Hypoxia Preconditioned Medium Protects A549 Lung Cancer Cells in a EPOR-dependent Fashion 18

5.5 Identification of Potential Factors Stimulating EPOR-mediated Radioresistance 20

6 **Discussion** 23

6.1 Outlook 28

6.2 Conclusion 28

7 **References** 30

8 **Acknowledgement** .....

9 **Curriculum Vitae** .....

1 Summary

Lung cancer is one of the most frequent causes for cancer related deaths worldwide and commonly treated with radio- and chemotherapy. However, resistant tumor cells often complicate therapeutic interventions. We analyzed if Erythropoietin receptor (EPOR) is involved in hypoxia-induced resistance to chemo- and radiotherapy in A549 lung cancer cells. A CRISPR/Cas engineered EPOR knock out cell line and shRNA EPOR knock down cells were used to investigate the response of EPOR deficient cells to radiation and Gemcitabine. We showed that EPOR expressing cells responded with resistance to chemo- and radiotherapy after hypoxia exposure. This effect disappears in EPOR deficient cells, suggesting that the loss of EPOR renders lung cancer cells more susceptible to the treatment, especially to irradiation. Radioresistance was independent of DNA damage, repair mechanisms and unexpectedly administration of Erythropoietin (EPO) as well as overexpression of endogenous EPO was not sufficient to mimic the hypoxia-induced EPOR-dependent radioresistance. However, hypoxic preconditioned medium of A549 cancer cells was able to protect normoxic A549 wild type cells, which express EPOR. By filtering the hypoxic preconditioned medium, we narrowed down the potentially cancer cell protective factor to a molecular weight larger than 100 kDa. First attempts to identify this factor suggest the possible involvement of extracellular vesicles and EFNB2, a factor thought to be associated with EPOR signaling.

2 Zusammenfassung

Lungenkrebs ist einer der häufigsten krebsbedingten Todesursachen weltweit und wird mit Chemo- und Radiotherapie behandelt. Oftmals treten jedoch Resistenzen auf, welche die Therapien erschweren.
Wir untersuchten ob und wie der Erythropoietin Rezeptor (EPOR) bei der Resistenz gegenüber Chemo- und Radiotherapie in A549 Lungenkrebs Zellen eine Rolle spielt. Mit einer CRISPR/Cas EPOR knock out Zelllinie, sowie mit shRNA EPOR knock down Zellen, konnten wir zeigen, dass EPOR exprimierende Zellen in Hypoxie eine Resistenz gegenüber Chemo- und Radiotherapie entwickeln, während Krebszellen ohne EPOR diese Fähigkeit verloren. Wir konnten zeigen, dass bei der EPOR abhängigen Resistenz weder bestrahlungs-induzierte DNA Schäden noch eine veränderte DNA Reparatur eine Rolle spielten.
Ausserdem wurde der Schutzeffekt, welcher unter Hypoxie induziert werden kann, durch blosse Zugabe von Erythropoietin (EPO), sowohl in Form von rekombinantem humanem EPO (rhEPO), als auch die endogene Überproduktion von EPO, unter normoxischen Bedingungen nicht erreicht. Allerdings war hypoxisch präkonditioniertes Medium von wild Typ und knock out Zellen in der Lage auch normoxische wild Typ Zellen, aber nicht EPOR knock out Zellen zu schützen, sogar nach herausfiltrieren aller Substanzen kleiner als 100 kDa aus dem präkonditionierten Medium. Erste Versuche zeigten, dass extrazelluläre Vesikel und der Faktor EFNB2, welcher mit den EPOR Signalwegen assoziiert sein könnte, eine Rolle spielen.

3 Introduction

Erythropoietin (EPO) is a glycoprotein mainly produced by the kidney in adults, which is essential for survival, differentiation and proliferation of erythroid progenitor cells [1] and therefore represents the major member of the group of erythropoiesis stimulation agents (ESA). It was originally thought to play an exclusive role for differentiation of red blood cells, stimulated by expression of hypoxia-inducible factor (HIF) under hypoxic conditions [2]. In addition it is reported to support angiogenesis as well as vasculogenesis [3]. In a variety of non-hematopoietic tissues like blood vessels, heart and peripheral nervous system, EPO can act as a strong cyto-protective factor [4, 5] [6] [7]. Recombinant human erythropoietin is clinically used to correct anemia predominantly in chronic kidney disease to restore red blood cell levels, but also to correct anemia in cancer patients where anemia correlates with poor patient survival [8, 9]. The only alternative treatment represents a repetitive red blood cell transfusion, which harbors the risk of infection, iron overload and thrombotic events [10]. Also cyclic hypoxia between treatments can occur, which was shown to be even more detrimental than acute hypoxia for tumor behavior [11]. If and how EPO impacts on tumors is still – even after decades of research – controversially discussed and many publications about the impact of recombinant human EPO treatment for cancer patients as well as for the impact in *in vitro* experiments seem to contradict each other. Several clinical studies could show an poor prognostic outcome for cancer patients undergoing rhEPO treatment [9], as well as growth response in in vitro and in vivo experiments [12] [13] [14]. Since the discovery of the erythropoietin receptor (EPOR) gene expression in various tumor cell lines and carcinomas [15], concerns about EPO-EPOR dependent survival and tumor progression were raised [16] [17]. It was discussed that rhEPO could influence cancer cells either by stimulating tumor angiogenesis by interacting with EPOR present on endothelial cells or direct via EPO/EPOR signaling on cancer cells, possibly inducing tumor proliferation, metastasis and resistance to therapeutic approaches [18] [1]. However, several other studies failed to demonstrate proliferative effects of exogenous rhEPO treatment in *in vitro* experiments as well as for prognostic outcome in patients [19] [20] [21]. Swift et al. found that even if EPOR was detectable on mRNA level in many cancer cell lines, no functional EPOR could be found in nearly all of the tested cell lines. In addition they could find no upregulation of EPOR in hypoxia, nor an activation of EPOR downstream pathways as

reaction to rhEPO administration [22]. These reports have led to precaution in prescribing rhEPO to anemic cancer patients, especially because mechanisms behind EPO/EPOR communication in cancer cells is not yet fully understood.

Because lung cancer is the leading cause of cancer death among both men and women, with 80% to 85% categorized as non-small cell lung cancer (NSCLC) [23][22] it represents an important subject for cancer related studies. NSCLC have been also reported to express EPOR [24] and it was shown that EPOR expression in NSCLC, results in decreased sensitivity to cisplantin in response to EPO stimulation [25]. Recently, it has been reported, that the co-expression of endogenous EPO-EPOR is a negative prognostic factor for early-stage non-small lung cancer (NSCLC) [26], and that co-expression is positively associated with poor survival of NSCLC patients [27]. In contrast Doleschel *et. al* could show in their study from 2015 that EPO administration in fact lead to a beneficial reaction to carboplantin, due to enhanced tumor vascularization and perfusion [24]. The important function of EPOR has been also shown in other types of cancer including glioma, where the loss of EPOR reduces cancer cell proliferation [28] and increases cancer cell response to irradiation and temozolomide [29]. Particularly hypoxic environment seems be an essential factor for resistance of cancer cells to radio and chemotherapy [30] [31]. EPO expression is induced under hypoxic conditions via hypoxia-inducible factor 2, which is a heterodimeric transcription factor consisting of a stably expressed β and an oxygen-regulated α-subunit. In conditions of low oxygen, HIF2-α escapes proteasomic degradation, followed by nuclear translocation and heterodimerization, finally leading to activation of target genes with EPO being one of them [32]. In this study we analyzed the role of EPOR in A549 lung cancer cells – especially in the context of hypoxia-induced resistance to chemo- and radiotherapy. We utilize shRNA knock down cells and the recently developed CRISPR/Cas technology to establish EPOR deficient A549 cells and explored their response to Gemcitabine, a first line lung cancer treatment as well as radiation, which is frequently used for lung cancer patients [23] [33] [34].

4 Materials and Methods

4.1 Cell culture

A549 adenocarcinomic human alveolar basal epithelial cells, used in this study were grown and maintained in Dulbecco's Minimum Essential Medium (MEM) with Earle's Salts supplemented with 2ml L-glutamine, 10% fetal bovine serum (heat inactivated), 20 mM HEPES buffer solution and Penicillin/Streptomycin. Cells were split twice a week in a ratio of 1/10 after washing cells with PBS and incubating them with Trypsin (Gibco by Life Technologies) for 2-3 minutes. A549 wild type cells, shRNA scrambled cells and shRNA EPO receptor knock down cells where a generous gift from Julian Aragonez [35] (Fig. 1A). Cells were kept at 37° C in normoxia (21% O2 and 5% CO_2) Revco (Thermo Scientific) or hypoxia at 37° C in normoxia (1% O2 and 5% CO_2) in Hera cell 240 incubator (Heraeus).

4.2 CRISPR/Cas9 EPOR Knock Out Generation

CRISPR/Cas9 knock out generation was done in cooperation with Julia Armbruster. To obtain EPOR KO cells with genetically identical wild type control, monoclonal colonies were generated by seeding single cells into 96 well plates growing them in MEM with 20% FCS. After expansion of monoclonal colonies protein was isolated analyzed for EPOR expression levels and clone number 9 was selected for CRISPR/Cas knock out, due to its high EPOR expression level. Two 20-nt long sgRNAs (Table 1) were designed using the CRISPR Design Tool [36] to follow the conventional (double strand break) and the Nickase approach of the CRISPR/Cas9 protocol [37].To briefly describe the process: pSpCas9(BB)-2A-Puro (PX459)(conventional double strand break) and pSpCas9n(BB)-2A-Puro V2.0 (PX462) (Nickase) were a gift from Feng Zhang (Addgene plasmid # 62988 # 62987) [38]. The vectors express Cas9 wild type (px459) or Cas9n (Nickase) (px462) of S.pyogenes and include the scaffold of sgRNA, as well as a Bbs1 cutting side to allow integration of sgRNA. Additionally, the vectors have an Ampicillin and a Puromycin resistance cassette allowing later selection of successfully transfected cells. The sgRNA 2 was cloned into the px459 Vector and, additionally, both sgRNA1 and sgRNA2 were cloned into two independent px462 vectors, expressing the mutated Cas9 enzyme with nickase activity (CRISPR/Cas9n) to guide Cas9 to target side. A549 cells were transfected with Lipofectamine 2000 according to the suppliers protocol [39] on 6-well plates using 2500 ηg plasmid DNA per well and transfected

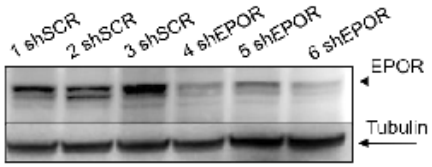
cells were selected with 1 µg/ml Puromycin (Sigma Aldrich). After selection monoclonal clones were generated as described above and genomic DNA was isolated from cell pellets with a homogenization buffer (50mM KCL, 10mM Tris-HCL (pH 8.3), 0.01mg/ml Gelatine, 0.045% Nonidet P-40, 0.045% Tween 20) with 50 µg/ml proteinase K (New England Biolabs) over night at 55° C. After heat inactivation of proteinase K at 95° C for 10 minutes, cells were centrifuged 5 min at 14860 rpm and 80 µl of the supernatant was transferred into a fresh tube. Genomic human EPOR DNA was amplified with a nested PCR using Primers shown in Table 1. PCR products were purified using QIAquick PCR purification kit (QIAGEN), cloned into pGEM-T vectors (Promega) and vectors were transformed into NEB 5-α competent E.coli (New England Biolaps) following the High Efficiency Transformation Protocol ([40] New England Biolaps). Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN) and sequenced by Microsynth (Fig. 1B). The DNA Sequences were analyzed using NCBI and resulting protein sequences were determined using the Expasy Translation tool (Fig. 1C)[41]. A549 EPOR knock out was validated via Western Blotting (Fig. 1D).

Table 1 Sequences of sgRNAs and EPOR primers to identify genetic modifications.

20 nt sgRNA	**Sequence**
Guide Sequence 1 (sgRNA1)	1_fwd: 5' **CACC**gATGATACAGCCCCCGCCACG 3' 1_rev: 3' cTACTATGTCGGGGGCGGTGC**CAAA** 5'
Guide Sequence 2 (sgRNA2)	2_fwd: 5' **CACC**gACCACCTCGGGGCGTCCCTC 3' 2_rev: 3' cTGGTGGAGCCCCGCAGGGAG**CAAA 5'**
EPOR Primer Nested PCR genomic DNA	**Sequence**
1. PCR	PCR 1_fwd : TCGGGGATCTGCCACTTAGA PCR 1_rev : CACCAAGTCAGCCCCCTTAG
2. PCR	PCR 2_fwd: GACCCAGCTGTGGACTGTG PCR 2_rev: TCCAGGACCCAGTCTAAGGG

Figure 1

A



B

WT human EPOR DNA sequence

5'...GGAGCCCTGGGCTCCCCGTGGCGGGGGCTGTATCATGGACCACCTCGGGGCGTCCCTCTGGCCCCAGGT...3'
3'...CCTCGGGACCCGAGGGGCACCGCCCCCGACATAGTACCTGGTGGAGCCCCGCAGGGAGACCGGGGTCCA...5'

Allele 1 (-9/+38=+29 base pairs)

5'...GGAGCCCTGGGCTCCCCGTGGCAGGGGCTGTA**GCCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCACCAA**
CCTCGGGGCGTCCCTCTGGCCCCAGGT...3'

Allele 2 (-5/+215=+210 base pairs)

5'...GGAGCCCTGGGCTCCCCGTGGCGGGGGCTGT**CAAATATGAAGGAATCATGGGAAATAGGCCCTCACATGTGA**
GCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCT
GACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATGAAGATACCAGGCGTTT
CCCCCTGGAAGCTCCCTCGTGCGCGGACCACCTCGGGGCGTCCCTCT...3'

C

WT human EPOR protein sequence

MDHLGASLWPQVGSLCLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTERLEDLVCFWEEAASAGVGPGNY
SFSYQLEDEPWKLCRLHQAPTARGAVRFWCSLPTADTSSFVPLELRVTAASGAPRYHRVIHINEVVLLDAPVGLVARLAD
ESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAW
SEPVSLLTPSDLDPLILTLSLILVVILVLLTVLALLSHRRALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSPC
TPFTEDPPASLEVLSERCWGTMQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDE
GSEASSCSSALASKPSPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGL
SDGPYSNPYENSLIPAAEPLPPSYVAC

Allele 1 (EPOR KO)

---(173)--- MTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLT
PSDLDPLILTLSLILVVILVLLTVLALLSHRRALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSPCTPFTEDP
PASLEVLSERCWGTMQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDEGSEASSC
SSALASKPSPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGLSDGPYSN
PYENSLIPAAEPLPPSYVACS

Allele 2 (EPOR KO)

---(173)--- MTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLT
PSDLDPLILTLSLILVVILVLLTVLALLSHRRALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSPCTPFTEDP
PASLEVLSERCWGTMQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDEGSEASSC
SSALASKPSPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGLSDGPYSN
PYENSLIPAAEPLPPSYVACS

D

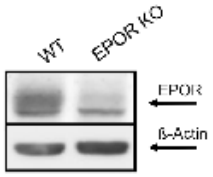


Fig.1 Establishing cell lines with different EPOR expression levels. **A:** EPOR expression levels in shRNA transfected monoclonal cells were confirmed via Western Blotting. Picture shows EPOR protein expression levels for 3 shSCR (ctrl) and 3 shEPOR (EPOR knock down). Tubulin was used as loading control. **B:** Wild type DNA Sequence of human EPOR gene is shown, followed by the modified 2 alleles of our CRISPR/Cas9 A549 EPOR knock out clone. Allele one shows a modification of 9 deleted base pairs (bp) with 38 inserted base pairs resulting in a prolongation of allele one for 29 bp (bold characters). Second allele was modified with 5 bp deletion and a 215 bp insertion resulting in a total prolongation of the allele for 210 bp (bold characters). **C:** Shows wild type human EPOR protein sequence with highlighted EPO-binding domain (bold). Translation of modified DNA sequences of the CRISPR/Cas knock out from panel B into protein sequences, indicates a loss of EPO binding domain (first 173 amino acids) on both alleles confirming a functional EPOR knock out. **D:** Verification of functional EPOR knock out on Protein level using Western Blot analysis. Beta actin was used as loading control.

4.3 Clonogenic Assay

Clonogenic Assays were performed on 6-well plates (for experiments with Gemcitabine treatment and EPO overexpression) in 2 ml 20% FCS MEM medium per well and cells were kept at normoxia (21% O_2) for 24 medium. 250 cells/well were seeded in three to four independent experiments (n) and each experiment was run in triplicates. For all other experiments, cells were seeded in duplicates for each individual experiment (n) in 25 cm^2 venti cap flasks (Corning, 430168) with 300 cells/flasks in 5ml 20% FCS MEM medium. 24 hours after seeding, cells were treated as described below and 48 hours after seeding, cells were exposed to a single dosage radiation (0,1,2,3 Gy) with a 6 megavolt (MV) linear accelerator (Clinac iX, Varian, Palo Alto, CA, USA) using photons. After radiation flasks or plates where placed back into normoxic or hypoxic incubators. All clonogenic Assays were fixed and stained after 7-10 days over night with a solution containing 0.5% Crystal-Violet and 0.6% glutaraldehyde. All colonies >50 cells were counted. All data was normalized to untreated control and results are shown in %.

Treatments:

a) Normoxic/ Hypoxic Clonogenic Assays

After seeding cells and incubating them for 24 hours in normoxia, flasks where either transferred into hypoxia or remained in normoxia. Another 24 hours later caps of hypoxic flasks where closed for irradiation outside of hypoxic incubators and reopened before being placed in hypoxia again.

b) Gemcitabine – a Chemotherapeutic often used as Treatment for NSCLC

Gemcitabine was added after 24 hours of incubation in normoxia and cells were either placed back into normoxia or transferred into hypoxic incubators.

c) rhEPO Treatment

24 hours after seeding, medium was removed and replaced with 5 ml of fresh 20% FCS MEM medium supplemented with 5 U/ml rhuEpo (Recormon; Roche).

d) Endogenous EPO Overexpression

A549 WT cells were seeded at 50% confluence in 6-well Plates and were transfected with Lipofectamine™ 3000 Transfection Reagent (Invitrogen) with 3.75 µl Lipofectamine 3000 per well and 2.5 µg total vector DNA/well, as recommended in

Lipofectamine 3000 Protocol [42]. PLenti6.3-hEpo, a gift from Juan Melero-Martin (Addgene plasmids # 50436), was used for endogenous EPO overexpression and an empty vector expressing mCherry (pmCherry-N1, Clontech, Cat. 632523) served as negative control as well as to verify transfection efficiency by immunofluorescence. Medium was replaced 24 hours after transfection and, after another 24 hours, collected. After centrifugation to remove cell debris, medium was supplemented with 10% and 2 ml were put on cells of a clonogenic assay.

e) WT and EPOR KO Preconditioned Medium

EPOR WT and KO cells were seeded incubated in 10 cm plates for 24 hours in 10% FCS Medium. Cells were then transferred to hypoxia or remained in normoxia for another 24 hours. Finally, medium was collected and pooled from all plates for each condition. PC medium was centrifuged to remove cell debris, filled into fresh tube and substituted with another 10% FCS. Afterwards, cells of clonogenic assays were incubated with 5 ml of PC Medium for 24 hours.

f) Centrifugal Filtration

Preconditioned (PC) medium was centrifuged to remove cellular debris, filled into fresh tubes and divided in two groups. One half of the medium remained at room temperature as a control to the further processed second half of PC medium. For further processing, PC medium was centrifuged in Centricon Plus-70 Centrifugal Filter Devices (Merck/Millipore; 3kDa, 30kDa, 100kDa) for 50 min at 21° C and 3500 x g to obtain concentrated medium and to remove molecules smaller than the indicated filter size. To recover the condensed PC medium, columns were inverted and centrifuged for 2 min at 1000 x g and the recovered PC medium was diluted in 40 ml fresh MEM medium without FCS. The process was repeated twice to ensure complete removal of all molecules smaller than the indicated filter size. At the final step, the condensed and filtered medium was diluted in fresh 20% FCS containing MEM medium and cells of clonogenic assays were incubated with 5 ml processed medium or unprocessed PC control medium.

g) Microvesicle Isolation (>100nm)

After 24 medium incubation 10% FCS preconditioned Medium (PC) of confluent hypoxic or normoxic 10 cm plates, was collected. Preconditioned (PC) Medium was

centrifuged to remove cell debris, filled into fresh tubes and divided in two groups. One half of the medium remained at room temperature as a control to the further processed second half of PC medium. For isolation of microvesicles > 100 nm in diameter from PC medium, different centrifugation steps where performed according to the following protocol [43]. The vesicle pellet was then taken up in original volume of fresh 20% FCS medium and cells of clonogenic assays were incubated with 5 ml processed medium or unprocessed control medium.

4.4 γH2AX – as Marker for DNA-damage

Round cover slips were placed in 24-well plates and sterilized with ethanol. After drying up, cells were seeded in these wells in 500 µl 10% FCS MEM medium (20000 cells/well). After 24 hours incubation in normoxia, cells were transferred into hypoxia or remained in normoxia. Another 24 hours later cells were either irradiated (1 and 3 Gy) or remained untreated. 0,5 or 24 hours post irradiation cells were washed with 1 x PBS and fixed with 4% Formalin for 15 minutes. After washing three times for 5 minutes in PBS, fixed cells were incubated with 0.5% T X-100 (T8787, Sigma Aldrich in PBS) for 30 minutes. After washing with PBS, cells were blocked for 1 hour with 3% BSA (A7906100G, Sigma Aldrich in PBS). γH2AX-antibody (γH2AX Ser139, Millipore, Temecula, CA, USA) was diluted 1:200 in 1% BSA and cells were incubated for 2 hours with the antibody solution. Afterwards, cells were washed 3 times with 1x PBS and exposed to the secondary antibody dilution (Alexa 488 donkey anti mouse (green) diluted 1:250 in PBS) for 1 hour. Finally, fixed cells were incubated with DAPI (blue, diluted 1:1000 in PBS) for 10 minutes, followed by 2 washing steps with PBS. The cover slips were mounted on one drop of Dako fluorescent mounting medium on glass slides and after short drying time, sealed with nail polish. Zeiss Imager 72 fluorescent microscope coupled to an 8-bit CCD camera was used to analyze slides. With 40x magnification, 2-4 visual fields pictures where taken until a minimum of 100 cells per condition per passage were captured (n=3-4) and DAPI-stained nuclei as well as γH2AX foci were manually quantified using Photoshop.

4.5 RNA Extraction, cDNA Synthesis and Real-time PCR

RNA was isolated using the ReliaPrep RNA Cell Miniprep System following the supplier instructions (Promega) and quantified using Nanodrop (Thermo Fisher). Isolated RNA was transformed to cDNA by using 10 µl of RNA (100 ng/ µl) and annealing it with 1 µl Oligo dT (10 µM; Promega) for 5 minutes at 65° C followed by rapid cooling on ice. After 15 µl of Master mix (5.57 µl H_2O, 5 µl 5x Reaction Buffer for RT (Thermo Fischer), 2.5 dNTPs (15 mM each, Thermo scientific), 1 µl RNasin (Promega), 0.25 µl 100x BSA (New England Biolaps), 0.5 µl Revert Aid Reverse Transcriptase (Thermo scientific)) was added to the RNA, samples were incubated at 42° C for 2 hours, followed by inactivation of the reaction at 65° C for 10 minutes. 175 µl H_2O were added to obtain a final cDNA concentration of 5 ng/µl. Primers were designed by Primer3 software [44] [45] and purchased at Microsynth. Used primers are shown in table 2. Master mix was prepared on ice containing 5 µl LightCycler 480 SYBR Green I Master (Roche Applied Science, Cat. 04707516001), 1 µl Primer forward (10 µM), 1 µl Primer reverse (10 µM) (Microsynth) and 1 µl H_2O per sample. Samples were run in duplicates and 2 µl cDNA (5ng/ µl) was added to 8 µl master mix per well, in MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems by life technologies) on ice. Plate was sealed with Optical Adhesive Covers (Applied Biosystems by Life Technologies), and centrifuged for 2-3 minutes at 2500 x g, before being placed in Thermocycler ABI7500 Fast (Applied Biosystems) to run Real-time PCR. Amplification profile was as follows: 50° C for 2 min; 95° C for 10 min; 40 cycles at 95° C a 30 sec and 60° C for 40 sec, followed by melt curve analysis. Fold changes were determined by delta-delta Ct method and normalized to beta-Actin.

Table 2 Primer sequences for genes that were analyzed with Real-time PCR (SYBR green method).

Target Gene	Primer Pair
ACTB NM_001101.2	Fwd: 5' CTGGAACGGTGAAGGTGACA 3' Rev: 5' AAGGGACTTCCTGTAACAACGCA 3'
EPO NM_000799.2	Fwd: 5' ATGTGGATAAAGCCGTCAGT 3' Rev: 5' AGTGATTGTTCGGAGTGGAG 3'
PTGES NM_004878.4	Fwd: 5' GGATGCCCTGAGACACGGAG 3' Rev: 5' CAGAAAGGAGTAGACGAAGCCCA 3'
MVP NM_005115.4	Fwd: 5' TCCAGAGGGTCCAGAAGGTCCGAGAGC 3' Rev: 5' GCCCCATCCCCAGCAGCCCAAAGG 3'
EphB4 NM_004444.4	Fwd: 5' ACAAACACGGACAGTATCTCATC 3' Rev: 5' GCACCAATCACCTCTTCAATCT 3'
EFNB2 NM_004093.3	Fwd: 5' CTCAACTGTGCCAAACCAGA 3' Rev: 5' GCCCTCCAAAGACCCATT 3'

4.6 Western Blot

Cells were lysed with RIPA Buffer (20 mM Tris, 150 mM Sodium chloride, 1% Triton X-100, 1% Na-deoxycholate, 0,1% SDS) and the Pierce Bicinchoninic Acid (BCA) Protein Assay was used for determination of protein concentration. Proteins were separated by SDS-PAGE and transferred to Nitrocellulose Blotting Membrane (GE Healthcare Life Science). After blocking the membrane in 5% milk in TBST (Rapidlait, Migros, Switzerland), the following primary Antibodies were diluted in 5% milk: rat anti-EPOR 1:200 (GM1201; Adlevon), rabbit anti-alpha Tubulin 1:1000 (ab4074; Abcam), mouse anti-ACTB 1:5000 (Sigma Aldrich). Blots were exposed to HRP conjugated secondary antibodies diluted in 5% milk in TBST: goat anti-rat 1:5000 (sc-2032; Santa Cruz), donkey-anti-rabbit 1:5000 (NA934V; Amersham), goat-anti-mouse 1:5000 (Santa Cruz sc-2031). Bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientifc) and recorded with FUJIFILM Intelligent Darkbox Las-300.

5 Results

5.1 The Loss of EPOR Sensitizes A549 Lung Cancer Cells towards Gemcitabine

Targeting EPOR in glioblastoma cells has been shown to increase the response to chemotherapeutics [29]. To study the impact of EPOR on chemotherapy in lung cancer, A549 cells with reduced (shRNA; knock down) and ablated (CRIPSR/Cas; knock out) EPOR expression levels were used. With exposure to increasing concentrations of Gemcitabine, the number of formed colonies in a clonogenic assay, as a measure of survival, declined in a dose-dependent manner (Fig. 2 A,B,D,E). No statistically significant difference in colony formation after Gemcitabine treatment between scrambled (shSCR) and knock down (shEPOR) clones under normoxic conditions was observed, although the results indicate a clear trend of higher gemcitabine sensitivity in shEPOR knock down clones (Fig. 2A). However, under hypoxic conditions shSCR clones are better protected from gemcitabine treatment and formed more colonies compared than shEPOR knock down clones (Fig. 2B).

Finally, A549 shSCR cells display a moderate but statistically not significant hypoxia-induced resistance to Gemcitabine treatment (Fig. 2C)[1].

In addition to shRNA mediated EPOR knock down, a CRISPR/Cas mediated EPOR knock out clone (EPOR KO) was generated to confirm these findings. In line with the shEPOR knock down experiments (Fig. 2A-C), functional ablation of EPOR increased the Gemcitabine sensitivity under hypoxia but not under normoxic conditions (Fig. 2D, E). As observed in shRNA knock down experiments, the exposure to hypoxia induced a moderate yet not significant resistance to Gemcitabine in EPOR WT clones but not in EPOR KO clones (Fig. 2F). These data suggest that the loss of EPOR sensitizes A549 lung cancer cells towards Gemcitabine. However, a hypoxia-inducible increase in chemo-resistance might only play a minor role.

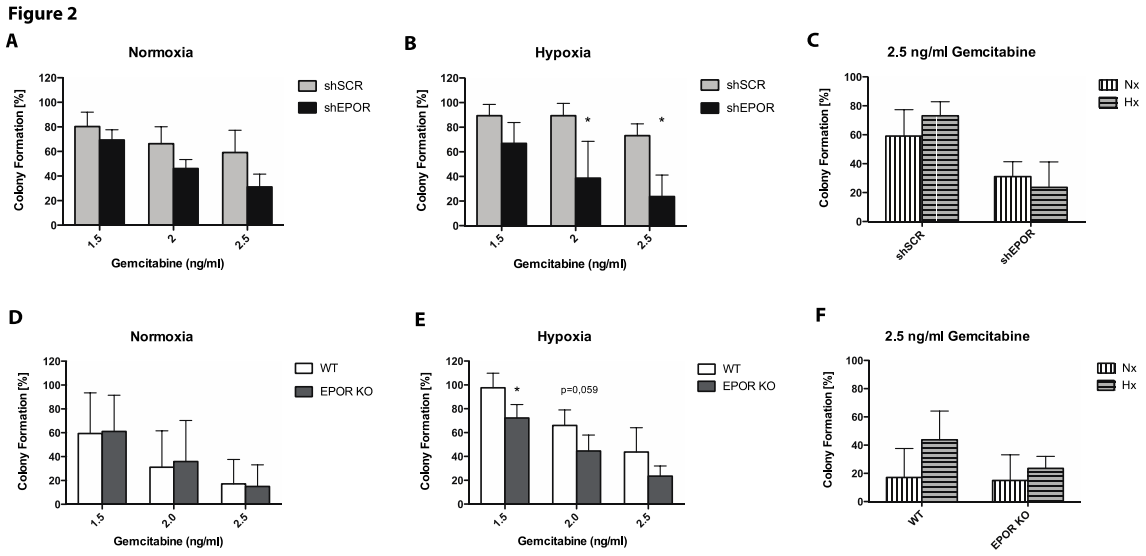


Fig. 2 EPOR dependent hypoxia-inducible resistance to Gemcitabine in A549 lung cancer cells

The role of EPOR in Gemcitabine efficacy in A549 lung cancer cells was analyzed by 3 individual shSCR control (light grey bars) and 3 shEPOR knock down (black bars) clones as well as by EPOR knock out (KO; CRISPR)(dark grey bars) and wild type (WT; white bars) clones.

A,B,C,D,E,F: Colony formation was analyzed by clonogenic assay under normoxic (21% O_2) and hypoxic (1% O_2) conditions with increasing concentrations of Gemcitabine treatment. Results are shown as colony formation in % of untreated controls (Y-axis). Cells were exposed to Gemcitabine 24 h post seeding and remained in normoxia or were transferred into hypoxia. Colonies were fixed, stained and quantified after 7-10 days. **A,B:** The response to increasing Gemcitabine concentrations (X-axis) of shSCR and shEPOR cells under normoxic (A) and hypoxic (B) conditions was analyzed. Data show significant differences between shSCR and shEPOR clones in hypoxia, but not in normoxia. **C:** Comparison of normoxia (horizontal stripes and hypoxia (vertical striped bars) exposed shSCR and shEPOR clones after treatment with 2.5 ng/ml Gemcitabine. Shown is colony formation (Y-axis) normalized to untreated shSCR and shEPOR knock down cells in %. **D,E:** Response to increasing Gemcitabine concentrations of WT (white bars) and EPOR knock out clones (dark grey bars) under normoxic (D) and hypoxic (E) conditions. **F:** Colony formation under normoxic (horizontal striped bars) and hypoxic (vertical striped bars) conditions of WT and EPOR KO cells after treatment with 2.5 ng/ml Gemcitabine. A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *p<0.05;).

[1] Please note: Results of figure 2 A-C were generated by Julia Armbruster as part of her master thesis [46]

5.2 Hypoxia-inducible Resistance to Radiotherapy is EPOR Dependent

It has been shown before that shEPOR knock down in U87 cells can increase the efficacy of irradiation, compared to shSCR (control) U87 cells [29].

To analyze how the expression of EPOR may impact the radiation efficacy in lung cancer, A549 lung cancer cells were exposed to different intensities of single radiation dosages (1; 2; 3 Gy) and cell survival was monitored with a clonogenic assay. With increasing dosages, the number of formed colonies declined in a dose-dependent manner. Comparing shSCR with shEPOR (Fig. 3A) as well as WT with EPOR KO in normoxia (Fig. 3B) reveals no differences between EPOR expressing and EPOR deficient groups in colony formation independent of the utilized dosage. In contrast, under hypoxic conditions shSCR cells form more colonies compared to shEPOR (Fig. 3C) reaching a statistical significance at a dosage of 2 Gy, suggesting that hypoxic exposure protects EPOR expressing cells but not EPOR deficient cells. Similarly, hypoxic EPOR KO cells show significantly less colonies after irradiation with 1 and 3 Gy than their respective WT controls (Fig. 3D). A different representation of shown data in Figure 3 E and F clearly demonstrates that 3 Gy exposed shSCR control (Fig. 3E) as well as EPOR WT cells (Fig. 3F) display a better survival of colonies under hypoxic conditions compared normoxia. In contrast, clones with reduced (shEPOR) (Fig. 3E) or ablated (EPOR KO) (Fig. 3F) EPOR gene expression levels fail to show the hypoxia-induced resistance to irradiation. These data support our hypothesis of EPOR being involved into hypoxia-induced resistance to radiation.

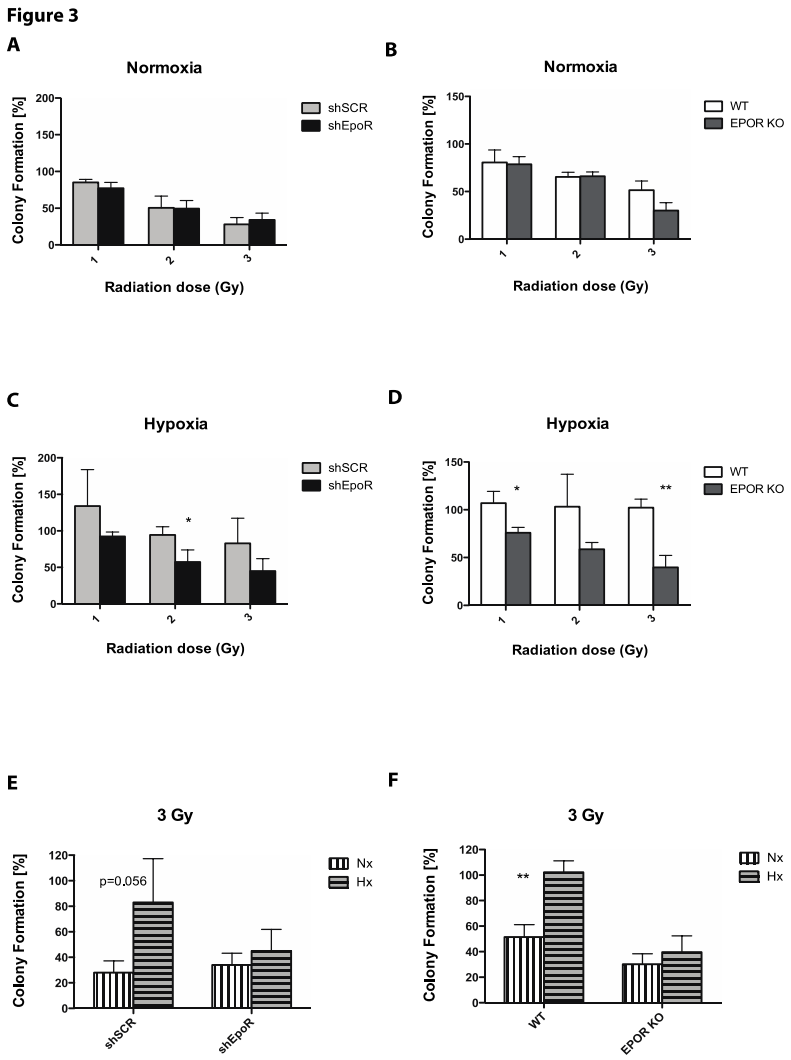


Fig. 3 EPOR dependent hypoxia-inducible resistance to radiotherapy in A549 lung cancer cells
Colony formation was analyzed after irradiation with clonogenic assay. Results where normalized to non-irradiated controls and are shown in % (Y-axis). Cells were seeded in normoxia (21% O_2) and either remained in normoxia or were transferred into hypoxia (1% O_2) 24 h prior to radiation with increasing radiation dosages. Colonies where fixed, stained and quantified after 7-10 days. **A,B:** Shown is the average response in normoxia of three individual shSCR control (light grey bars) and shEPOR knock down clones (black bars) (A) as well as of the WT (white bars) and EPOR KO (dark grey bars) clone (B) to increasing dosages of radiation (X-axis). **C,D:** Shown is the average response in hypoxia of three individual shSCR control and shEPOR knock down clones (C) as well as of the WT and EPOR KO clone (D) to increasing dosages of radiation (X-axis). **E,F:** Comparison of normoxia (horizontal striped bars) and hypoxia (vertical striped bars) exposed shSCR and shEpoR clones after irradiation (3 Gy) (E), as well as of WT and EPOR clones (F). A Students t-test was performed for statistics. (Mean ± SD; n=3; *p<0.05; **p<0.01)

5.3 EPOR-dependent Radioresistance does not Depend on Protection from DNA-damage or Repair of DNA-damage

It is known that radioresistance in hypoxia is associated with decreased DNA-damage due to a decreased fixation of potentially lethal DNA double stand breaks, as well as activation of additional DNA-repair pathways [47].

To explore if EPOR dependent radioresistance involves protection from DNA damage or DNA repair mechanisms, γH2AX was used as a measure of DNA-damage (Fig. 4A and B). Immunofluorescence staining of γH2AX under normoxic conditions (Fig. 4C) suggests that DNA damage in non-irradiated EPOR WT and KO cells (baseline) is comparable. Radiation with 1 Gy leads to a significantly higher number of γH2AX foci/cell after 30 min compared to baseline, but no difference between EPOR WT and KO cells was observed (Fig. 4C). Similarly, hypoxia exposed cells display neither differences in non-exposed cells (baseline) nor 30 minutes after irradiation (Fig. 4D). Despite the identical γH2AX response of WT and KO clones, we further observed, surprisingly, no significant difference between normoxic and hypoxic incubated WT cells (Fig. 4E) as well as EPOR KO cells (4F), suggesting that hypoxia-induced radioresistance might be independent of differences in DNA damage in our experimental setup. Whereas γH2AX quantification early after irradiation (e.g. 30 minutes post irradiation) may reflect the intensity of DNA damage, γH2AX at a later second time point (e.g. 24 h) represents a mixture of DNA damage and DNA repair. To assess if DNA repair mechanisms might be involved in EPOR-dependent radioresistance, γH2AX was quantified by immunostaining 24 h after radiation in normoxia and hypoxia exposed EPOR WT and KO cells (Fig. 4 G, H). However, 24 hours after radiation with a dosage of 3 Gy, no differences in DNA-damage between EPOR KO and WT cells in response to radiation under normoxia (Fig. 4G) or hypoxia (Fig. 4H) were detected. Furthermore, the γH2AX baseline value of EPOR WT and KO clones and under normoxic as well as hypoxic conditions was identical in non-irradiated cells (Fig. 4G,H). Finally, no difference in DNA-damage of each clone can be seen in between normoxia or hypoxia neither for WT cells (Fig. 4I) nor EPOR KO cells (Fig. 4 J), suggesting that hypoxia-induced radioresistance might be also independent of DNA repair mechanisms in our experiments.

In conclusion, these data show that irradiation induces DNA damage in A549 cancer cells, but indicate that EPOR-dependent radioresistance may not involve DNA damage or DNA repair mechanisms. Moreover, because γH2AX is not altered by hypoxia exposure in irradiated and non-irradiated EPOR WT cells it appears that hypoxia-induced radioresistance may not require protection from DNA damage nor on DNA repair (Fig.4 E,F,I,J) in our experimental setup.

Figure 4

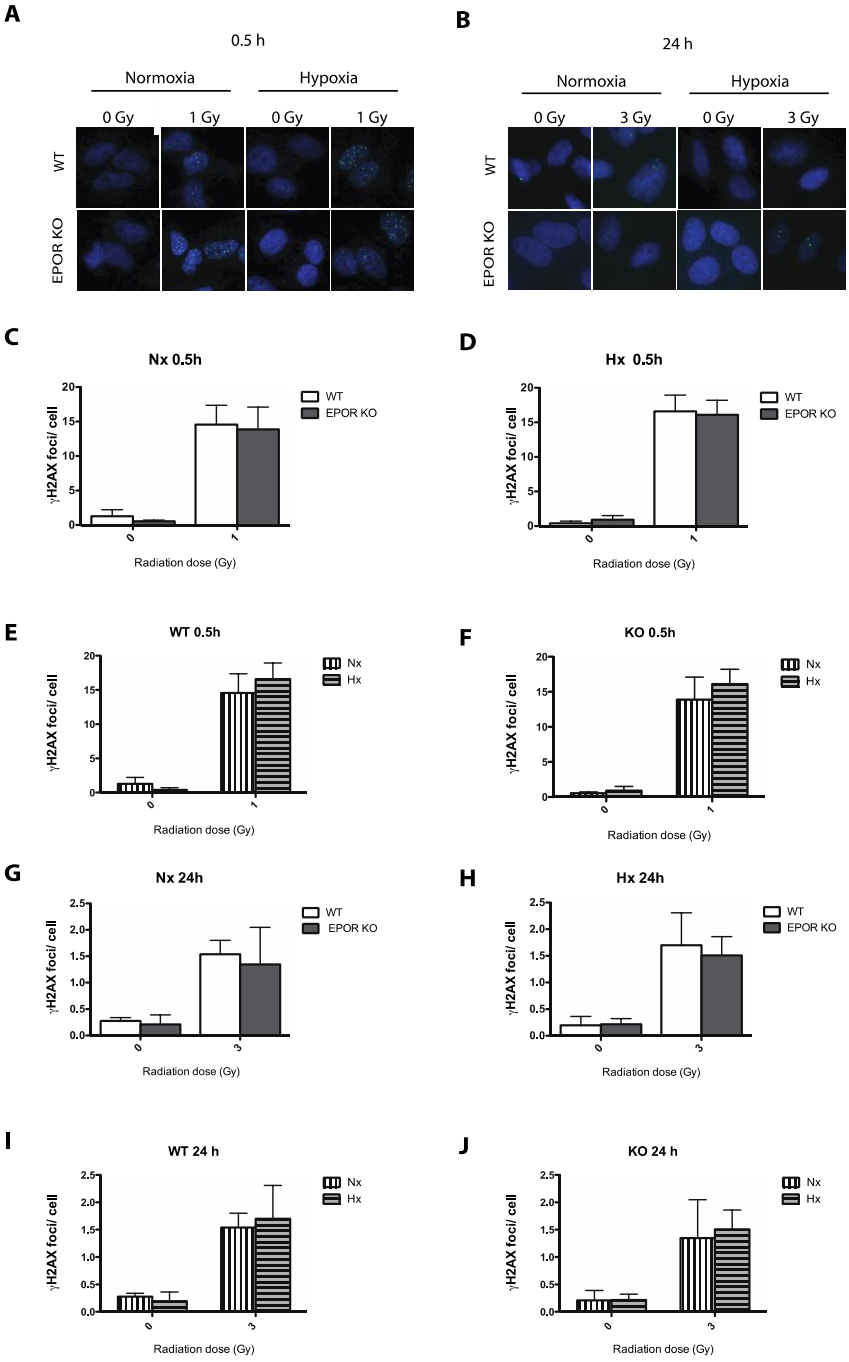


Fig. 4 DNA Damage and repair mechanisms are not controlled by EPOR under both, normoxic and hypoxic conditions
DNA Damage of A549 WT and EPOR KO clones after irradiation was visualized and quantified using a γH2AX Antibody (Anti-phospho-Histone H2AX (Ser139) Antibody, (#05-636), Milipore). Cells were seeded under normoxic conditions (21% O_2) and exposed to either normoxia or hypoxia (1% O_2) 24 h prior to radiation. After fixation, cells where stained for γ-H2AX (green) and counterstained with DAPI (blue). At least 100 cells were counted and average γH2AX-foci per nucleus where calculated. **A,B:** Representative pictures of γH2AX staining 0.5 h after radiation dosage of 1 Gy (A) or 24 h after irradiation with 3 Gy (B) **C,D:** Shown is the average number of γH2AX-foci/cell of normoxic (B) and hypoxic (C) EPOR KO and WT cells without irradiation (0 Gy) as well as 30 min after radiation with 1 Gy. **E,F:** Alternative representation of the data from C and D to visualize the impact of hypoxia exposure on DNA damage. Average number of γH2AX-foci/cell in WT (E) and EPOR KO (F) cells to contrast normoxia (horizontal striped bars) with hypoxia (vertical striped bar) **G,H:** Shown is the average number of γH2AX-foci/cell of normoxic (C) and hypoxic (D) EPOR KO and WT cells without irradiation (0 Gy) as well as 24 h after radiation with 3 Gy. **I,J:** Alternative representation of the data from G and H to visualize the impact of hypoxia exposure on DNA damage/repair. Average number of γH2AX-foci/cell in WT (I) and EPOR KO (J) cells to contrast normoxia (horizontal striped bars) with hypoxia (vertical striped bar) No significant differences

between WT and EPOR KO could be seen at either time points or conditions. A Students t-test was performed for statistics. (Mean ± SD; n=3-4)

5.4 Hypoxia Preconditioned Medium Protects A549 Lung Cancer Cells in a EPOR-dependent Fashion

The data above indicate that hypoxia-induced radioresistance requires the expression of EPOR in A549 cancer cells suggesting that cancer cell-specific EPO expression, which might be induced during hypoxia exposure, is responsible for the protective effect. To determine if hypoxia-inducible protection is mediated via auto- or paracrine pathways, experiments with normoxia and hypoxia preconditioned medium were performed. Medium of hypoxia but not normoxia exposed A549 EPOR WT cells protected A549 EPOR WT but not EPOR KO cells in a colony forming assay after irradiation with 3 Gy (Fig. 5 A), suggesting that the incubation of colony forming cells with hypoxia preconditioned medium is sufficient to mediate radioresistance without any need to directly expose these cells to hypoxia. In other words, hypoxia preconditioning causes the release of a factor into the medium protecting cells in a paracrine way, which have been kept in normoxia during the whole experiment. Furthermore, hypoxia preconditioned medium of A549 EPOR KO cells is also able to protect A549 EPOR WT but not KO colonies (Fig. 5B) indicating that that the release of such a factor is independent of EPOR but requires EPOR to confer radioresistance. EPO is the classical ligand for the EPO receptor and the most plausible candidate for EPOR-dependent radioresistance in A549 cells. However, EPO mRNA expression was hardly expressed in normoxic (CT >35) and not up regulated in hypoxia-exposed A549 cancer cells (data not shown). Furthermore, normoxic A549 EPOR WT cells, which become radioresistant when exposed to hypoxia-preconditioned medium, were not protected from 3 Gy irradiation after the treatment with a high dose of rhEPO (5 U/ml) (Fig. 5C). The activity of rhEPO has been verified in mice where it induced hematopoiesis (data not shown). To assess if the synthetic origin of rhEPO may cause this ineffectiveness, cells were also transfected with an EPO overexpressing Vector to analyze the impact of preconditioned medium with endogenously produced EPO on radioresistance of A549 EPOR WT cells (Fig. 5D). Compared to a mCherry control vector, the EPO expression vector induced EPO mRNA expression levels >4000 fold (average n=3) (Fig. 5E). However, even the strong EPO overexpression caused no protection of A549 EPOR WT cells after 3 Gy irradiation, suggesting that EPO itself is not required to mediate EPOR-dependent radioresistance.

Figure 5

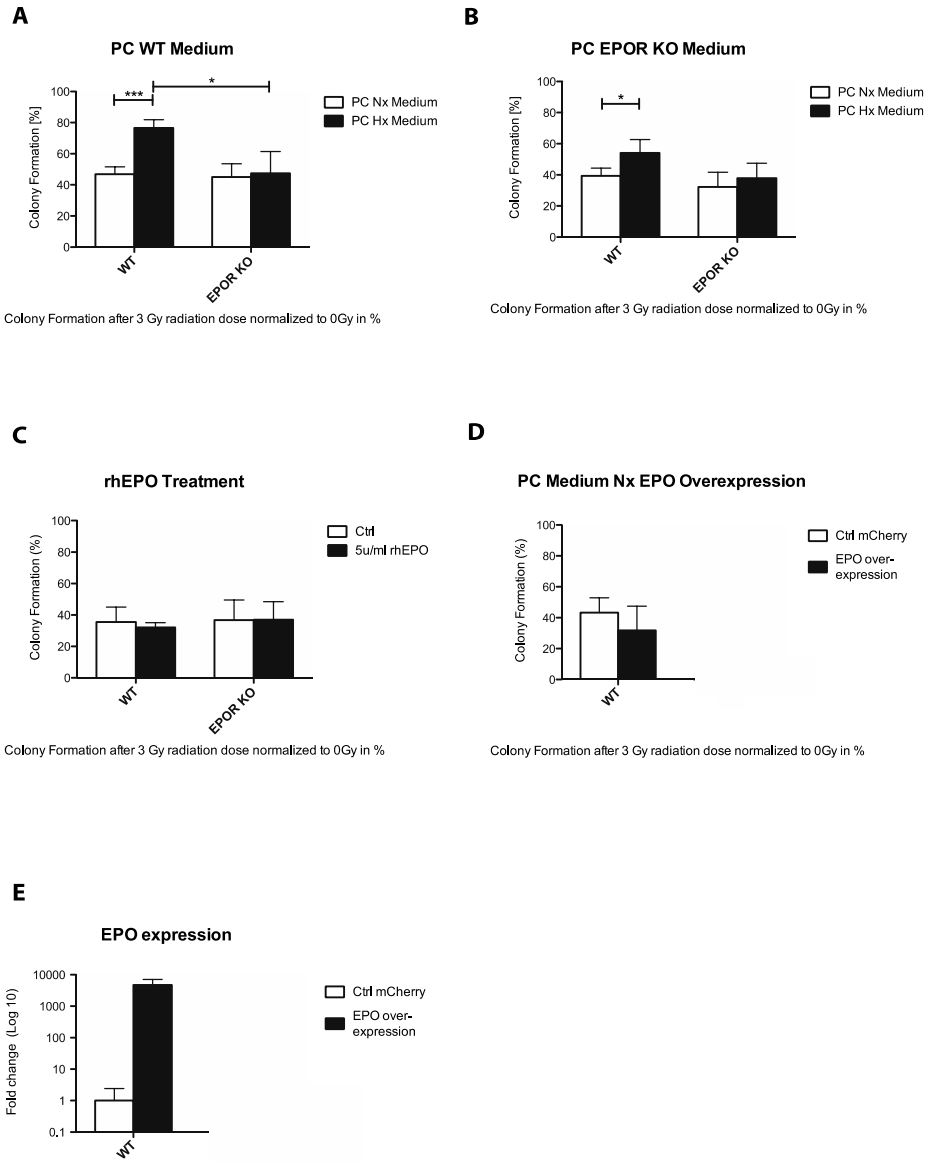


Fig. 5 Hypoxia-induced radioresistance depends on EPOR but might be EPO independent
Colony formation was analyzed after a single dose of radiation (3 Gy) via clonogenic assay. Data were normalized to non-irradiated controls and are shown in % (Y-axis). **A,B:** Medium was taken from confluent WT (A) or EPOR KO cells (B) under normoxic (21% O_2, white bars) or hypoxic (1% O_2, black bars) conditions to obtain preconditioned medium (PC). PC medium of WT (A) or EPOR KO cells (B) was added to WT and EPOR KO clonogenic assays one day prior to irradiation treatment (3 Gy) and colony formation was analyzed 7-10 days later. Hypoxic PC medium (black bars) of WT cells (A) and EPOR KO cells (B) results in an increased number of WT colonies but not EPOR KO colonies, when compared to normoxic PC medium. **C:** 24 h prior to irradiation either fresh medium (white bars) or fresh medium supplemented with 5 U/ml rhEPO (black bars) was added to cells and colony formation was analyzed 7-10 days later. Neither WT not EPOR KO cells display a difference after EPO treatment. **D:** 24 h prior to irradiation either medium of mCherry transfected WT cells (white bars) or of EPO overexpressing transfected WT cells (black bars) was added to cells and colony formation was analyzed 7-10 days later. **E:** Semi-quantitative analysis of mRNA by SYBR Green Real-time PCR was used to analyze EPO gene expression of transfected A549 WT cells (X-axis). White bars represent mCherry-transfected cells, whereas black bars represent cells transfected with an EPO overexpressing Vector (PLenti6.3-hEpo).Target gene expression was normalized to β-Actin and is shown as fold changes (Y-axis) (delta-delta Ct method). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *p<0.05; **p<0.01 ***p<0.001)

5.5 Identification of Potential Factors Stimulating EPOR-mediated Radioresistance

Since hypoxia-induced radioresistance occurred to be not solely based on EPO, additional factors with cyto-protective properties and a history of association with the EPO/EPOR signaling pathway were considered. Possible candidates, either as alternative ligands or as co-stimulating (together with EPO) factors, were analyzed by Real-time PCR. Prostaglandin E Synthase (PTGES) is a novel HIF-inducible gene [48] regulating Prostaglandin E_2 (PGE_2) biosynthesis, which itself is reported to enhance EPO-mediated STAT5 transcriptional activity [49]. Therefore, we quantified Prostaglandin E Synthase expression levels (Fig. 6A). However, no significant differences between WT and EPOR KO clone or between normoxia and hypoxia were observed, although there is a clear trend for PTGES to be downregulated in hypoxia. However, hypoxia preconditioned medium most likely requires an upregulated and released factor. Consequently, we did not further analyze PTGES.

The Major vault Protein (MVP) in humans also called “drug-resistance related protein” (LRP) is a 110 kDa in size, which is known to stand in relation with drug resistance in cancer cells [50] and interacts with pathways also interacting with EPO. Even though WT cells show a higher expression of MVP then EPOR KO cells, again we found a downregulation in hypoxia for both cell lines (Fig. 6B), excluding MVP as a potential candidate.

Recently it has been shown that EPO acts as an alternative ligand for the Ephrin B4 receptor (EphB4)[51]. However, it is not clear if EphrinB2 (EFNB2), the classical ligand for EphB4, is also able to bind and activate EPOR. The expression pattern of EphB4 (Fig. 6C) and EFNB2 (Fig. 6D) was analyzed in normoxia and hypoxia exposed A549 EPOR WT and KO cells. EphB4 expression displays no differences in both, A549 EPOR WT and KO cells under normoxic and hypoxic conditions. In contrast, EFNB2 was induced during hypoxia in EPOR WT (2-fold) and KO cells (2-fold). Moreover, comparing EFNB2 expression levels between EPOR WT and KO cells indicates that the lack of EPOR increases the expression of EFNB2 by 3 to 4-fold under both, normoxic and hypoxic conditions. If EFNB2 is and alternative ligand for EPOR in A549 cancer cells, the induction of EFNB2 in EPOR deficient cells might be a compensatory mechanism.

To verify if EFNB2, with a size of 36 kDa is a potential candidate that mediates radioresistance via EPOR, centrifugal filtration with different filter sizes was performed. Centrifugal filtration removed all molecules smaller than 3 kDa, 30 kDa and 100 kDa from the normoxia and hypoxia preconditioned medium of A549 EPOR WT cells. Filtering

hypoxia-preconditioned medium with filter-sizes of 3 and 30 kDa, did not reduce the protective effect of hypoxia preconditioned medium (data not shown). Even after filtering preconditioned medium with a 100 kDa centrifugal filter (Fig. 6E), colony formation in A549 EPOR WT but not KO cells treated with filtered medium was increased after 3 Gy irradiation. Since most of receptor ligands are smaller than 100 kDa we performed an extracellular vesicle centrifugation to isolate microvesicles out of normoxic and hypoxic WT PC medium and to analyze their role in hypoxia-induced radioresistance (Fig. 6F). However, using a simplified protocol to isolate these vesicles caused no protection of A549 WT cancer cells from irradiation. These preliminary data suggests that the factor released during hypoxia conferring resistance to radiotherapy, might be indeed bigger than 100 kDa.

Figure 6

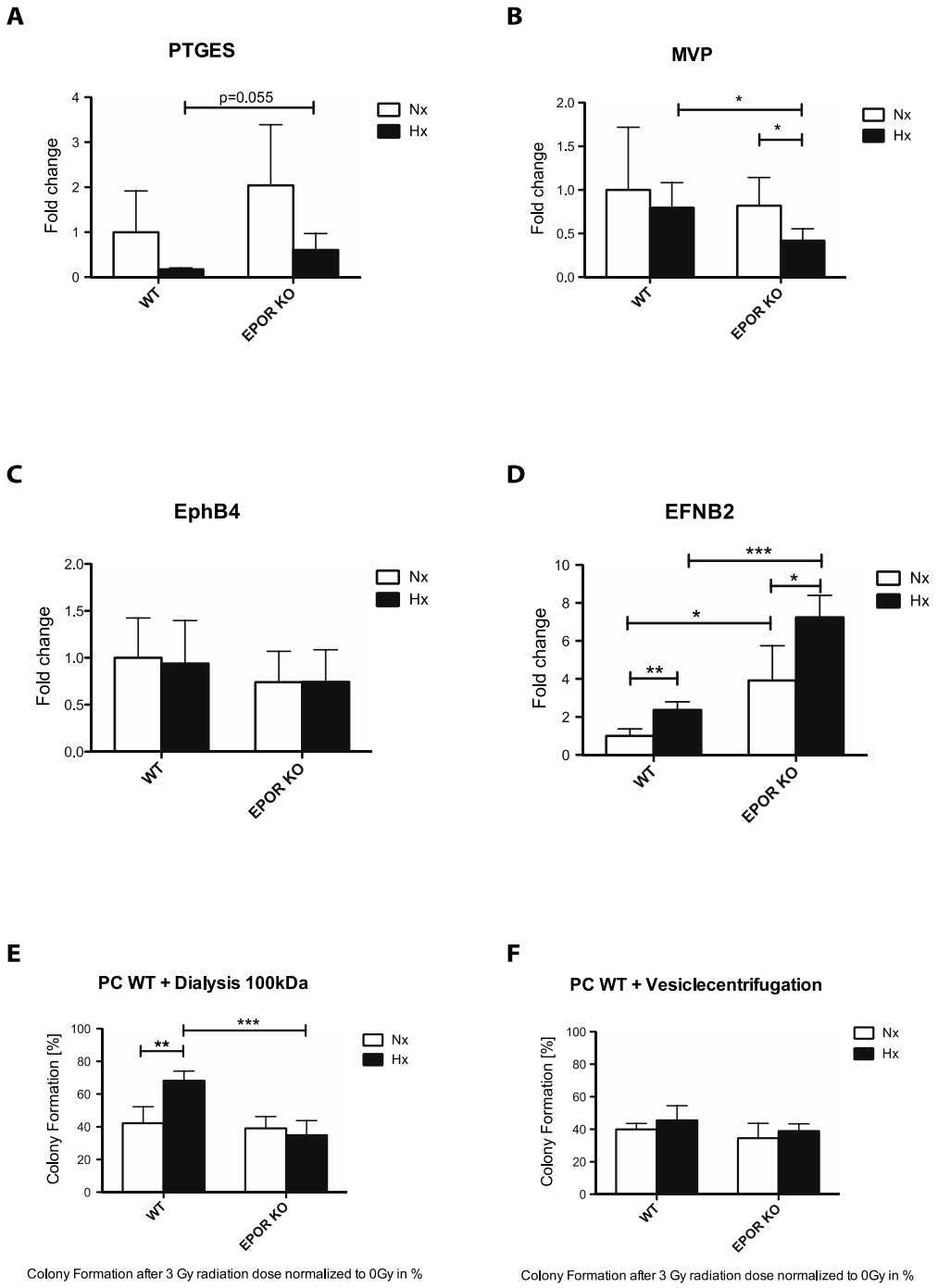


Fig. 6 Possible cross interactions for EPO-EPOR signaling

A,B,C,D: Semi-quantitative analysis of mRNA by SYBR Green real time PCR was used to analyze gene expression of A549 WT and EPOR KO clones (X-axis). Cells were exposed to hypoxia (black bars; 1% O_2) or remained in normoxia (white bars; 21% O_2) for 24 h, RNA was isolated and cDNA prepared. Target gene expression was normalized to β-Actin and is shown as fold changes (Y-axis) (delta-delta Ct method). Analyzed Genes are Prostaglandin E synthase (A) (PTGES), Major Vault Protein (MVP) (B), Ephrin receptor B4 (EphB4) (C) as well as Ephrin B2 (EFNB2) (D). **E,F:** Colony formation was analyzed after a single dose of radiation (3 Gy), via clonogenic assay. Data were normalized to non-irradiated control in % (Y-axis). Medium was preconditioned on WT cells for 24 h on confluent plates in either normoxic (21% O_2; white bars) or hypoxic (1% O_2; black bars) conditions. **E:** Preconditioned medium (PC) was filtered by 100 kDa centrifugal filtration to remove all substances smaller than 100 kDa. WT and EPOR KO cells were treated with filtered, preconditioned medium 24h before X-ray treatment (3 Gy). Filtered hypoxia preconditioned medium protects WT but not EPOR KO cells from radiation. **F:** PC medium was filtered to isolate microvesicles, and microvesicle pellet was then taken up in Medium and distributed on

cells 24h before X-ray treatment (3 Gy). A Students t-test was performed for statistics. (Mean ±SD; n=3-5; *p<0.05, **p<0.01, ***p<0.001)

6 Discussion

In this study, we showed that A549 lung cancer cells express EPOR and generated A549 EPO receptor knock out cells with CRISPR/Cas that were used together with previously generated A549 shRNA EPOR knock down cells to analyze the role of EPOR in chemo- and radioresistance. We demonstrate that hypoxia-inducible chemo- and radioresistance of EPOR expressing A549 cells disappears in cells with reduced or completely ablated EPOR expression levels. These data indicate that EPOR is indeed involved in resistance to chemo- and radiotherapy – especially under hypoxic conditions. However, neither rhEPO administration nor EPO overexpression protect A549 cells from chemo- and radiotherapy, whereas hypoxic-preconditioned medium is sufficient to protect these cells under normoxic conditions, suggesting that EPO is at least not the only factor required to protect A549 lung cancer cells from chemo- and radiotherapy.

We and others [35] showed that A549 human lung cancer cells express EPOR similar to a large number of other human cancer cells [12, 24, 52]. Several studies report that EPOR is also expressed in different types of tumors in human cancer patients [53-55]. However, the clinical relevance and the underlying mechanisms are not yet fully understood [14, 26]. Recently, He *et. al.* showed that elevated EPO/EPOR protein levels in NSCLC patients correlated with poor survival [27]. A549 human lung cancer cells (and other cell lines) display radio- and chemotherapy resistance – especially under hypoxic conditions [56] [30] [31]. With the genetic ablation of EPOR expression using shRNA and CRISPR/Cas technology we showed that hypoxia-inducible resistance to radiation and Gemcitabine is mediated by EPOR, i.e. targeting EPOR results in cancer cells that remain susceptible to radiation or Gemcitabine even under hypoxic conditions. Similarly, previous studies reported a decreased radio- and chemoresistance in U87 EPOR silenced glioma cells [29], thus supporting our recent findings in A549 lung cancer cells. Interestingly, EPOR inhibition in U87 also seems to sensitize glioblastoma cells to irradiation and Temozolomide under normoxic conditions, associated with increased DNA Damage in shEPOR cells. Reduced DNA damage and increased DNA repair are well-studied mechanisms that account especially for radioresistance [47]. In contrast to U87 glioma cells, EPOR deficient A549 lung cancer cells are neither more susceptible to radiation under normoxic conditions nor was an

involvement of DNA damage and repair mechanism under normoxia and hypoxia observed. It has to be mentioned that time points and irradiation dosages between our study and Peres *et al.* [29] differed, which might explain different results. However, it seems to be more likely that A549 lung cancer cells utilize other mechanisms and pathways downstream of EPOR to mediate hypoxia-induced resistance to chemo- and radiotherapy – one of them might be a reduced level of apoptosis [57]. Consequently, it might be possible that tumors in human patients, that express EPOR, might involve different, tumor-specific pathways to protect cancer cells from therapeutic interventions.

EPO is the ligand for the EPO receptor and has been reported to negatively influence clinical outcome of cancer patients [9, 58-60]. Thus, it seems obvious that EPOR mediated resistance to irradiation under hypoxia involves EPOR activation by EPO. Indeed, several studies report on increased proliferation in breast and lung cancer cells after rhEPO administration [61] [62]. In the present study, we showed that hypoxia-induced radioresistance requires the expression of EPOR. However, treating A549 cells with rhEPO instead of exposing them to hypoxia failed to protect them from irradiation. EPO overexpression, to test if these cells might only respond to endogenously produced but not recombinant EPO, had no protective effect either after irradiation. Similarly, Rheinbothe *et. al*., showed that the partial loss of EPOR in human breast cancer cell lines including MDA-MB-231 and MCF7 results in reduced cell proliferation, whereas the administration of rhEPO did not further increase cancer cell proliferation [63]. The interpretation of these data seems to be easy, suggesting that EPO might be not required for EPOR-dependent cancer cell resistance. However, two aspects show possible limitations and have to be considered:

I) Many cells including A549 cancer cells require cell culture medium supplemented with fetal calf serum (FCS), which contains bovine EPO. Human and bovine EPO show a high similarity (79% analogic sequence via gene blast), suggesting that bovine EPO might be able to activate human EPOR. It is currently not known if bovine EPO can bind the human EPOR. However, it has been reported that U87 glioma and HT 100 primary cervical cancer cells only respond to rhEPO when FCS was heat inactivated suggesting that bovine EPO indeed may activate human EPOR [56]. All experiments conducted in this study were performed with heat inactivated FCS, but it cannot be ruled out that, although heat inactivated, FCS might still contain biologically active bovine EPO. Given the high amount of FCS used in cell culture, the heat-inactivated

bovine FCS might be sufficient to fully activate EPOR, explaining the non-responsiveness of A549 cells to EPO administration and overexpression.

II) Exposure to hypoxia not only regulates the release of potentially cytoprotective factors but also intracellularly signaling pathways like apoptosis [64] and cell cycle[65]. Additionally, hypoxia might be required to upregulate expression and activation of EPOR[14]. However, A549 cells did not display altered EPOR mRNA gene expression when exposed to hypoxia. To analyze if A549 lung cancer cells require direct exposure to hypoxia to induce radioresistant processes, we utilized preconditioned (PC) medium of hypoxia exposed A549 wild type cells and showed that hypoxic PC medium is sufficient to protect EPOR-expressing but not EPOR-deficient A549 cells from irradiation under normoxic conditions. Of note, hypoxic PC medium of EPOR deficient A549 cells also protects EPOR-expressing but not EPOR-deficient A549 cells, suggesting that the released factor that mediates radioresistance in A549 wild type cells is regulated independently of EPOR.

EPO has been shown to affect cells independent of the EPO Receptor [66], probably via the ephrin receptor B4 (EphB4) [51] . On the other hand EPO has been also shown to have no effect on proliferation in EPOR positive cells [63, 67]. Our data suggest that radioresistance under hypoxia requires EPOR but if EPO is required remains questionable, leading us to two hypotheses; first it is possible that bovine EPO in the FCS – although heat-inactivated – completely activates EPOR under both normoxic and hypoxic conditions and neither administration of rhEPO nor endogenous EPO expression is required to activate EPOR. To confer radioresistance the fully activated EPOR requires a second, hypoxia-inducible and secreted factor that binds to its receptor. Only the co-stimulation of both sufficiently induces radioresistance under hypoxia. A second possibility is that EPOR has an alternative ligand. In conclusion, our data indicate that a factor either replacing or in addition to EPO is involved in mediating hypoxia-induced radioresistance in an EPOR dependent fashion (Fig.7).

Figure 7

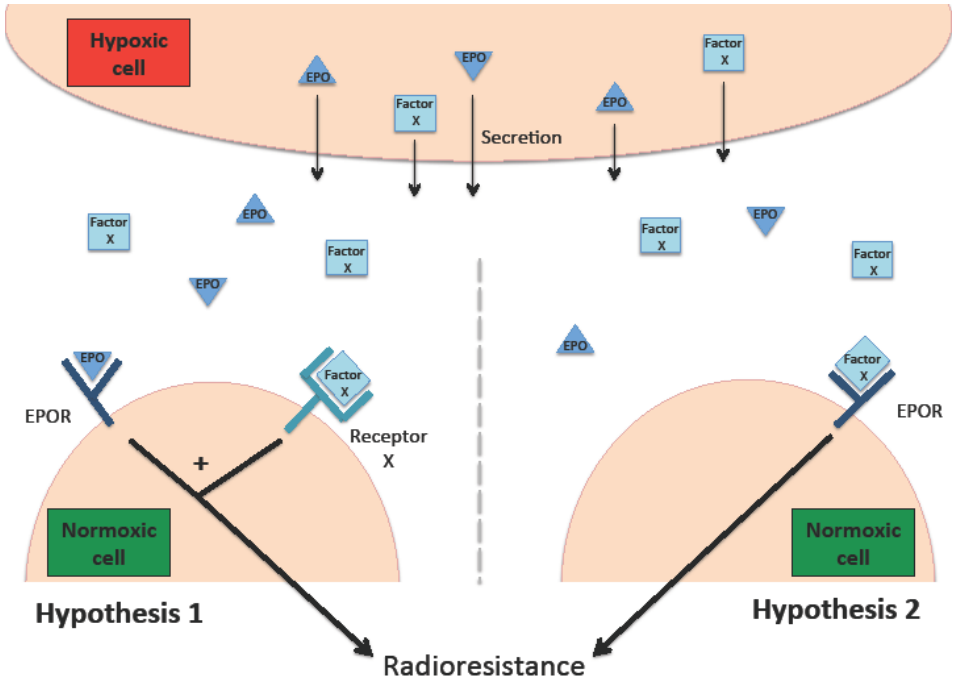


Fig. 7 Possible mechanisms for hypoxia-induced radioresistance
Hypoxic A549 cells secret a so far unidentified factor (here named as factor X), independent of EPOR expression. The incubation with hypoxic preconditioned medium renders EPOR expressing cells resistant to radiotherapy, suggesting a paracrine pathway. Currently, two theories could explain this mechanism. **Hypothesis 1:** EPO is binding to its receptor EPOR, but a second stimulus (co-stimulation theory), here presented as Factor X, binds to its respective Receptor X is needed to cause hypoxia-induced radioresistance. **Hypothesis 2:** EPOR presenting cells develop hypoxia-induced resistance by Factor X binding to EPOR as an alternative ligand instead of EPO.

Currently, no other ligand but EPO has been discovered to bind EPOR. However, it has been recently reported, that EPO alternatively binds to EphB4, besides its original ligand Ephrin B2 (EFNB2) [51]. Moreover, the authors demonstrated that EphB4 but not EPOR correlated with poor outcome of EPO treated cancer patients. Given that EPO indeed binds to EphB4 it might be also possible that EFNB2 is also able to bind EPOR to replace EPO as ligand. In fact, we observed that EFNB2 mRNA expression is induced in hypoxia-exposed cells. Moreover, cells lacking EPOR appeared to have a higher baseline expression of EFNB2 suggesting that cells might try to compensate the loss of EPOR. It has further been proposed that there might be an EPOR-EPHB4 heterodimer or an EPOR-b(CR)-EPHB4 heterotrimer [68]. We found that EPHB4 is expressed in normoxia as well as hypoxia-exposed A549 cells suggesting that hypoxia-inducible EFNB2 might be indeed an alternative EPOR ligand or is at least required to confer radioresistance. In addition to that we analyzed mRNA expression of other factors that have been previously reported to enhance EPO signaling. For example, erythroid cells display enhanced colony formation after EPO administration, if Prostaglandin-

E_2 is used as a co-administered substance [49]. However, we observed no differential gene expression of Prostaglandin E Synthase (PTGES) expression in hypoxia-exposed cells.
To reduce the number of potential, secreted factors that account for hypoxia-inducible radioresistance, centrifugal filter units with different pore sizes where used to narrow down the molecular weight of this factor. We observed that the protective factor remained in a fraction larger than 100 kDa, which would exclude EFNB2 with its size of ~56 kDa as a candidate. One candidate protein that has been reported to be associated with EPO/EPOR and its downstream signaling pathway JAK/STAT is the mayor vault protein (MVP) [48] [69], also known as resistant related protein (LRP) with a size of ~110 kDa. It seems to play a role for resistance to anti-cancer therapy [50] by clearing drugs from their subcellular targets through sequestration into exocytotic vesicles [70]. Although we observed no changes in DNA damage and repair in our model, MVP has been also shown to support DNA-repair as an intracellular transport protein or shuttling vector for DNA-repair related Proteins [71]. However, A549 cells did not respond with increased MVP mRNA expression levels during hypoxia, suggesting that it rather plays no role in EPOR-mediated and hypoxia-induced radioresistance.
Since the majority of receptor binding ligands is smaller than 100 kDa, we further explored the possibility of extracellular vesicles instead of single proteins being involved in EPOR-mediated radioresistance. Extracellular vesicles are larger than 100 kDa and divide into two groups of small endosomal-derived exosomes (diameter <100 nm) and membrane-derived microvesicles (> 100nm) [43]. Such vesicles have been reported to play critical roles in cancer progression, dissemination and therapy [72-74] and carry proteins, mRNA, microRNA and membrane bound receptors or other molecules [75, 76] that can be either taken up by target cells or directly interact with their target cells [76, 77]. Aforementioned EFNB2 with a molecular size of ~56 kDa has been shown to be present in such vesicles [78] and could protect A549 cells from radiation, if it is indeed associated to such vesicles that are larger than 100 kDa. To analyze if microvesicles, released during hypoxia, are capable of mediating EPOR-dependent radioresistance, we performed a pilot experiment and showed that the microvesicles fraction, which we isolated (>100 kDa) did not protect A549 cells from irradiation. However, it has to be mentioned that conventional FCS, that is typically used in cell culture contains already a large amount of such vesicles [76] and our simplified isolation protocol of vesicles depended solely on medium centrifugation and may mask the protective effect. These experiments should and will be repeated with vesicle free FCS and isolation of

bioactive vesicles (microvesicles and exosomes) will be performed with optimized approaches and protocols.

6.1 Outlook

To ultimately identify the factor that mediates EPOR-dependent radioresistance, several experiments will be performed in the future: Improved isolation methods of extracellular vesicles and especially exosomes that are smaller than 100 nm will be applied, to test if vesicle secretion plays a role for the in hypoxia-induced resistance and if EFNB2, as a component of these vesicles, is involved in that mechanism. A second candidate to analyze is the stem cell factor (SCF), which also has been reported to be present in extracellular vesicles [79], as well as to exist in a membrane bound isoform, possibly leading to vesicular secretion [80]. It has been reported that SCF gene expression is induced under hypoxic conditions [81] and that blocking SCF is sufficient to inhibit cancer stem cells proliferation and survival promoted by chemotherapy in NSCLC [82] or leading to increased colony formation and migration [83]. In addition functional and compositional characterization of extracellular vesicles, human recombinant EFNB2 and SCF protein will be used, to analyze if their administration protects normoxic cells from radioresistance in an EPOR-dependent fashion. Moreover, it is essential to understand if EPOR-mediated radioresistance involves the classical EPO/EPOR/JAK2 pathway, which will be tested by utilizing AG490, a well established JAK2 inhibitor to target specifically the JAK2/STAT pathway [84, 85], and soluble EPOR will be used to sequester bovine and human EPO from the cell medium.

6.2 Conclusion

Our results show that EPOR may have indeed a clinical relevance for lung cancer, even though expressed to very low levels. Our current data imply that EPO administration to anemic cancer patients might be without effects for therapy resistance if EPOR in lung cancer cells binds an alternative ligand or if lung cancer cells with low EPOR expression levels are already saturated by endogenously produced EPO. If these hypotheses hold true, cancer-specific targeting EPOR instead of restricting EPO administration could not only prevent potentially EPO induced cancer promotion but might be a possible anti cancer strategy to prevent therapy resistance especially under hypoxic conditions because tumor hypoxia often

causes therapy resistance in many different kinds of tumors [86]. However, before that it is important to fully decipher and understand the underlying mechanisms and the role of EPOR in cancer. At the current stage of research and in the light of the current manuscript (A549 EPOR/EPO) it is still mandatory to carefully use EPO in cancer patients.

7 References

1. Wang, L., L. Di, and C.T. Noguchi, *Erythropoietin, a novel versatile player regulating energy metabolism beyond the erythroid system.* Int J Biol Sci, 2014. **10**(8): p. 921-39.
2. Song, X., et al., *Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene.* Cancer Chemother Pharmacol, 2006. **58**(6): p. 776-84.
3. Muller-Ehmsen, J., et al., *Role of erythropoietin for angiogenesis and vasculogenesis: from embryonic development through adulthood.* Am J Physiol Heart Circ Physiol, 2006. **290**(1): p. H331-40.
4. Jelkmann, W., et al., *The erythropoietin receptor in normal and cancer tissues.* Crit Rev Oncol Hematol, 2008. **67**(1): p. 39-61.
5. Chong, Z.Z., J.Q. Kang, and K. Maiese, *Erythropoietin: cytoprotection in vascular and neuronal cells.* Curr Drug Targets Cardiovasc Haematol Disord, 2003. **3**(2): p. 141-54.
6. Grimm, C., et al., *HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration.* Nat Med, 2002. **8**(7): p. 718-24.
7. Sasaki, R., S. Masuda, and M. Nagao, *Erythropoietin: multiple physiological functions and regulation of biosynthesis.* Biosci Biotechnol Biochem, 2000. **64**(9): p. 1775-93.
8. Caro, J.J., et al., *Anemia as an independent prognostic factor for survival in patients with cancer: a systemic, quantitative review.* Cancer, 2001. **91**(12): p. 2214-21.
9. Temkin, S.M., et al., *Erythropoietin administration during primary treatment for locally advanced cervical carcinoma is associated with poor response to radiation.* Int J Gynecol Cancer, 2006. **16**(5): p. 1855-61.
10. Khorana, A.A., et al., *Blood transfusions, thrombosis, and mortality in hospitalized patients with cancer.* Arch Intern Med, 2008. **168**(21): p. 2377-81.
11. Nowrousian, M.R., *Recombinant Human Erythropoietin (rhEPO) in Clinical Oncology2008*. 2008: Springer Wien New York.
12. Westenfelder, C. and R.L. Baranowski, *Erythropoietin stimulates proliferation of human renal carcinoma cells.* Kidney Int, 2000. **58**(2): p. 647-57.
13. Feldman, L., et al., *Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells.* Prostate, 2006. **66**(2): p. 135-45.
14. Acs, G., et al., *Erythropoietin and erythropoietin receptor expression in human cancer.* Cancer Res, 2001. **61**(9): p. 3561-5.
15. Fandrey, J., *Erythropoietin receptors on tumor cells: what do they mean?* Oncologist, 2008. **13 Suppl 3**: p. 16-20.
16. Lappin, T.R., A.P. Maxwell, and P.G. Johnston, *Warning flags for erythropoiesis-stimulating agents and cancer-associated anemia.* Oncologist, 2007. **12**(4): p. 362-5.
17. Sytkowski, A.J., *Does erythropoietin have a dark side? Epo signaling and cancer cells.* Sci STKE, 2007. **2007**(395): p. pe38.
18. Hardee, M.E., et al., *Erythropoietin biology in cancer.* Clin Cancer Res, 2006. **12**(2): p. 332-9.
19. Liu, W.M., et al., *Effect of haemopoietic growth factors on cancer cell lines and their role in chemosensitivity.* Oncogene, 2004. **23**(4): p. 981-90.
20. Laugsch, M., et al., *Lack of functional erythropoietin receptors of cancer cell lines.* Int J Cancer, 2008. **122**(5): p. 1005-11.
21. Jeong, J.Y., et al., *Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells.* Int J Cancer, 2008. **122**(2): p. 274-80.

22. Swift, S., et al., *Absence of functional EpoR expression in human tumor cell lines.* Blood, 2010. **115**(21): p. 4254-63.
23. https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html.
24. Doleschel, D., et al., *Erythropoietin improves the accumulation and therapeutic effects of carboplatin by enhancing tumor vascularization and perfusion.* Theranostics, 2015. **5**(8): p. 905-18.
25. Merkle, R., *Impact of growth factors, therapeutic inhibitors and cytostatic compounds on the response of non-small-cell lung carcinoma cell lines* 2015.
26. Saintigny, P., et al., *Erythropoietin and erythropoietin receptor coexpression is associated with poor survival in stage I non-small cell lung cancer.* Clin Cancer Res, 2007. **13**(16): p. 4825-31.
27. Lei. *Local blockae of self-sustainable erythropoietin signaling supresses tumor progression in non-small lung cancer*. 2017.
28. Peres, E.A., et al., *Targeting the erythropoietin receptor on glioma cells reduces tumour growth.* Exp Cell Res, 2011. **317**(16): p. 2321-32.
29. Peres, E.A., et al., *Silencing erythropoietin receptor on glioma cells reinforces efficacy of temozolomide and X-rays through senescence and mitotic catastrophe.* Oncotarget, 2015. **6**(4): p. 2101-19.
30. Chapman, J.D., et al., *Measuring hypoxia and predicting tumor radioresistance with nuclear medicine assays.* Radiother Oncol, 1998. **46**(3): p. 229-37.
31. Murray, D., et al., *Influence of oxygen on the radiosensitivity of human glioma cell lines.* Am J Clin Oncol, 2003. **26**(5): p. e169-77.
32. Rankin, E.B., et al., *Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo.* J Clin Invest, 2007. **117**(4): p. 1068-77.
33. Denlinger, C.E., B.K. Rundall, and D.R. Jones, *Proteasome inhibition sensitizes non-small cell lung cancer to histone deacetylase inhibitor-induced apoptosis through the generation of reactive oxygen species.* J Thorac Cardiovasc Surg, 2004. **128**(5): p. 740-8.
34. cancer.org, https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html.
35. Maxwell, P., et al., *Novel antibodies directed against the human erythropoietin receptor: creating a basis for clinical implementation.* Br J Haematol, 2015. **168**(3): p. 429-42.
36. http://tools.genome-engineering.org.
37. Cong, L. and F. Zhang, *Genome engineering using CRISPR-Cas9 system.* Methods Mol Biol, 2015. **1239**: p. 197-217.
38. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system.* Nat Protoc, 2013. **8**(11): p. 2281-2308.
39. https://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_2000_Reag_protocol.pdf.
40. https://www.neb.com/protocols/1/01/01/high-efficiency-transformation-protocol-c2987.
41. http://web.expasy.org/translate/.
42. https://tools.thermofisher.com/content/sfs/manuals/lipofectamine3000_protocol.pdf.
43. Menck, K., et al., *Isolation and Characterization of Microvesicles from Peripheral Blood.* J Vis Exp, 2017(119).
44. Koressaar, T. and M. Remm, *Enhancements and modifications of primer design program Primer3.* Bioinformatics, 2007. **23**(10): p. 1289-91.

45. Untergasser, A., et al., *Primer3--new capabilities and interfaces.* Nucleic Acids Res, 2012. **40**(15): p. e115.
46. Armbruster, J., *Erythropoietin Receptor and Cancer- The role of EpoR in cancer cell growth, mitochondrial respiration ad chemotherapy resistance*, in *Veterinary Physiology*. 2016, University of Zurich.
47. Bristow, R.G. and R.P. Hill, *Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability.* Nat Rev Cancer, 2008. **8**(3): p. 180-92.
48. Lee, J.J., et al., *Hypoxia activates the cyclooxygenase-2-prostaglandin E synthase axis.* Carcinogenesis, 2010. **31**(3): p. 427-34.
49. Boer, A.K., et al., *Prostaglandin-E2 enhances EPO-mediated STAT5 transcriptional activity by serine phosphorylation of CREB.* Blood, 2002. **100**(2): p. 467-73.
50. Banerjee Dixit, A., et al., *Upregulation of breast cancer resistance protein and major vault protein in drug resistant epilepsy.* Seizure, 2017. **47**: p. 9-12.
51. Pradeep, S., et al., *Erythropoietin Stimulates Tumor Growth via EphB4.* Cancer Cell, 2015. **28**(5): p. 610-22.
52. Selzer, E., et al., *Erythropoietin receptor expression in human melanoma cells.* Melanoma Res, 2000. **10**(5): p. 421-6.
53. Mohyeldin, A., et al., *Survival and invasiveness of astrocytomas promoted by erythropoietin.* J Neurosurg, 2007. **106**(2): p. 338-50.
54. Miller, C.P., N. Urban, and C.A. Blau, *Quantitative comparison of erythropoietin receptor levels in the epithelial versus endothelial fractions of primary breast tumors.* Anticancer Res, 2011. **31**(4): p. 1189-95.
55. Aapro, M., et al., *Effects of erythropoietin receptors and erythropoiesis-stimulating agents on disease progression in cancer.* Br J Cancer, 2012. **106**(7): p. 1249-58.
56. Belenkov, A.I., et al., *Erythropoietin induces cancer cell resistance to ionizing radiation and to cisplatin.* Mol Cancer Ther, 2004. **3**(12): p. 1525-32.
57. Zou, Y.R., et al., *Erythropoietin Receptor Activation Protects the Kidney From Ischemia/Reperfusion-Induced Apoptosis by Activating ERK/p53 Signal Pathway.* Transplant Proc, 2016. **48**(1): p. 217-21.
58. Untch, M., et al., *PREPARE trial: a randomized phase III trial comparing preoperative, dose-dense, dose-intensified chemotherapy with epirubicin, paclitaxel and CMF versus a standard-dosed epirubicin/cyclophosphamide followed by paclitaxel +/- darbepoetin alfa in primary breast cancer--results at the time of surgery.* Ann Oncol, 2011. **22**(9): p. 1988-98.
59. Untch, M., et al., *PREPARE trial: a randomized phase III trial comparing preoperative, dose-dense, dose-intensified chemotherapy with epirubicin, paclitaxel, and CMF versus a standard-dosed epirubicin-cyclophosphamide followed by paclitaxel with or without darbepoetin alfa in primary breast cancer--outcome on prognosis.* Ann Oncol, 2011. **22**(9): p. 1999-2006.
60. Gascon, P., et al., *Effects of CERA (continuous erythropoietin receptor activator) in patients with advanced non-small-cell lung cancer (NSCLC) receiving chemotherapy: results of a phase II study.* Ann Oncol, 2010. **21**(10): p. 2029-39.
61. Chan, K.K., et al., *Erythropoietin drives breast cancer progression by activation of its receptor EPOR.* Oncotarget, 2017. **8**(24): p. 38251-38263.
62. Paul, I., et al., *Pre-operative plasma erythropoietin concentration and survival following surgery for non-small cell lung cancer.* Lung Cancer, 2006. **51**(3): p. 329-34.
63. Reinbothe, S., et al., *EPO-independent functional EPO receptor in breast cancer enhances estrogen receptor activity and promotes cell proliferation.* Biochem Biophys Res Commun, 2014. **445**(1): p. 163-9.

64. Sermeus, A., et al., *Hypoxia-induced modulation of apoptosis and BCL-2 family proteins in different cancer cell types.* PLoS One, 2012. **7**(11): p. e47519.
65. Goda, N., et al., *Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia.* Mol Cell Biol, 2003. **23**(1): p. 359-69.
66. Okazaki, T., et al., *Erythropoietin promotes the growth of tumors lacking its receptor and decreases survival of tumor-bearing mice by enhancing angiogenesis.* Neoplasia, 2008. **10**(9): p. 932-9.
67. Belda-Iniesta, C., et al., *Human recombinant erythropoietin does not promote cancer growth in presence of functional receptors expressed in cancer cells.* Cancer Biol Ther, 2007. **6**(10): p. 1600-5.
68. Debeljak, N., P. Solar, and A.J. Sytkowski, *Erythropoietin and cancer: the unintended consequences of anemia correction.* Front Immunol, 2014. **5**: p. 563.
69. Steiner, E., et al., *The major vault protein is responsive to and interferes with interferon-gamma-mediated STAT1 signals.* J Cell Sci, 2006. **119**(Pt 3): p. 459-69.
70. Mossink, M.H., et al., *Vaults: a ribonucleoprotein particle involved in drug resistance?* Oncogene, 2003. **22**(47): p. 7458-67.
71. Lara, P.C., et al., *MVP and vaults: a role in the radiation response.* Radiat Oncol, 2011. **6**: p. 148.
72. Ginestra, A., et al., *The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness.* Anticancer Res, 1998. **18**(5A): p. 3433-7.
73. Ginestra, A., et al., *Membrane vesicles in ovarian cancer fluids: a new potential marker.* Anticancer Res, 1999. **19**(4C): p. 3439-45.
74. Muralidharan-Chari, V., et al., *Microvesicles: mediators of extracellular communication during cancer progression.* J Cell Sci, 2010. **123**(Pt 10): p. 1603-11.
75. Abreu, S.C., D.J. Weiss, and P.R. Rocco, *Extracellular vesicles derived from mesenchymal stromal cells: a therapeutic option in respiratory diseases?* Stem Cell Res Ther, 2016. **7**(1): p. 53.
76. Ochieng, J., et al., *Anchorage-independent growth of breast carcinoma cells is mediated by serum exosomes.* Exp Cell Res, 2009. **315**(11): p. 1875-88.
77. Camussi, G., et al., *Exosomes/microvesicles as a mechanism of cell-to-cell communication.* Kidney Int, 2010. **78**(9): p. 838-48.
78. Tauro, B.J., et al., *Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes.* Methods, 2012. **56**(2): p. 293-304.
79. Quesenberry, P.J., et al., *Role of extracellular RNA-carrying vesicles in cell differentiation and reprogramming.* Stem Cell Res Ther, 2015. **6**: p. 153.
80. Slanicka Krieger, M., et al., *The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow.* Exp Hematol, 1998. **26**(5): p. 365-73.
81. Han, Z.B., et al., *Hypoxia-inducible factor (HIF)-1 alpha directly enhances the transcriptional activity of stem cell factor (SCF) in response to hypoxia and epidermal growth factor (EGF).* Carcinogenesis, 2008. **29**(10): p. 1853-61.
82. Levina, V., et al., *Elimination of human lung cancer stem cells through targeting of the stem cell factor-c-kit autocrine signaling loop.* Cancer Res, 2010. **70**(1): p. 338-46.
83. Wu, H., et al., *Functional interaction of erythropoietin and stem cell factor receptors is essential for erythroid colony formation.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1806-10.

84. Miyamoto, N., et al., *The JAK2 inhibitor AG490 predominantly abrogates the growth of human B-precursor leukemic cells with 11q23 translocation or Philadelphia chromosome.* Leukemia, 2001. **15**(11): p. 1758-68.
85. Samanta, A.K., et al., *Janus kinase 2: a critical target in chronic myelogenous leukemia.* Cancer Res, 2006. **66**(13): p. 6468-72.
86. Gray, L.H., et al., *The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy.* Br J Radiol, 1953. **26**(312): p. 638-48.

8 Acknowledgement

First of all I would like to thank my supervisor Dr. Markus Thiersch, for two wonderful years as his first doctoral student. You did a great job with teaching and supervising and still always left room for own ideas and humorously working atmosphere. Thank you for the possibility to learn so much about research and scientific work.
Also I want to thank Prof. Dr. Max Gassmann for giving me the opportunity to do my research and to write my thesis in his group, Prof. Dr. Carla Rohrer Bley for cooperating with us for irradiation treatments and for being my coreferent and Dr. Hassan Chaachouay for his cosupervision.
A big thank you to the whole Gassmann Group, I will miss working with you. I want to thank especially Julia Armbruster and Hyrije Ademi for helping me whenever I needed help -private or work related. Because of you I enjoyed coming to work every single day. Thank you for being friends for life.

I want to thank the “Marie-Louise von Muralt Stiftung”, the “Swiss National Foundation” and the “Stiftung für wissenschaftliche Forschung” for their financial support. This project would not have been possible without it.

Last but not least I would like to thank my family, particularly my parents Cathleen Jänicke and Thilo Siebert, as well as my boyfriend Marco von Büren, for always being there, supporting me and believing in me. I want to thank my friend Christina Keck, who was the biggest support I could wish for, during our studies and for life.

9 Curriculum Vitae

Vorname Name	Nadine Jänicke
Geburtsdatum	11.04.1989
Geburtsort	Berlin, Deutschland
Nationalität	Deutsch

Schulausbildung

08/1999-06/2008	Gymnasium der Stadt Würselen , Deutschland

Höchster Schulabschluss

13/06/2008	Abitur, Gymnasium der Stadt Würselen , Deutschland

Studium

10/2009- 06/2015	Veterinärmedizin, Ludwig Maximilians Universität, München, Deutschland

Abschlussprüfung vet. med.

11/06/2015	Ludwig Maximilians Universität, München, Deutschland

Anfertigung der Dissertation

06/2015 – 09/2017	unter Leitung von Prof. Dr. Max Gassmann, Direktor des Instituts für Veterinärphysiologie Vetsuisse-Fakultät Universität Zürich

Alle fachrelevanten Anstellungen nach Abschluss des veterinärmedizinischen Studiums

06/2015 – 09/2017	Doktorat, Institut für Veterinärphysiologie Vetsuisse-Fakultät Universität Zürich